CFP and YFP photostabilities are differentially affected by common mounting fluids

To the Editor:

Cyan and yellow fluorescent proteins (CFP and YFP, respectively) are very frequently used in a great variety of experiments employing fluorescence microscopy, because they can be distinguished quite easily by appropriate filter sets. Especially the original mammalian optimized versions, also known as enhanced cyan and yellow fluorescent proteins (ECFP and EYFP) are commonly applied, as they have been among the first spectrally distinct variants of green fluorescent protein[1]. Moreover, they are well suited for fluorescence resonance energy transfer (FRET) microscopy to visualize protein interactions or conformational changes[2]. In that case, the energy of the donor fluorophore (CFP) is transferred to an acceptor fluorophore (YFP) by a dipole interaction resulting in a decrease of donor and an increase in acceptor fluorescence. This phenomenon can be visualized by various microscopy techniques. One of them is based on the increase of donor fluorescence after photobleaching of the acceptor, which eliminates the energy transfer. This is practically done by acquiring a donor (CFP) image, followed by bleaching of the acceptor (YFP) with its specific excitation wavelength and the acquisition of a second donor image[3]. An increase in donor fluorescence, which is usually visualized by calculating a ratio or a difference image of the two donor images, indicates close proximity of FRET donor and acceptor proteins. When we applied this technique to cells transfected with interacting CFP- and YFP-tagged proteins or a positive CFP-YFP FRET probe, we noticed a striking difference in results obtained with living cells and those generated with fixed cells after mounting them on coverslips with a commercial mounting fluid (Fig. 1A). While the donor increase was perfectly visible in living cells, we did not observe a significant increase in fixed cells. Moreover, we noticed that the donor fluorescence faded much faster in the mounted, fixed samples, while it appeared more difficult to bleach the acceptor. This prompted us to determine the kinetics of photobleaching of CFP and YFP in live versus fixed cells and to investigate the influence of the fluid, in which the cells are embedded for microscopy. After mounting the cells in a commercial mounting fluid (Dako Fluorescence Mounting Medium or Thermo Scientific Ultramount), the bleaching of CFP was much faster than in live cells at the same illumination intensity (Fig. 1B, upper panel), whereas the bleaching of YFP was slowed down (Fig. 1B, middle panel). Interestingly, the kinetics of bleaching hardly differed between live cells imaged in medium and cells that had been fixed with 4% paraformaldehyde and imaged in PBS (Suppl. Fig. 1). This was observed for both CFP and YFP. Mounting of fixed cells in PBS/glycerol (1:9)

resulted in enhanced photostability of CFP, while bleaching kinetics of YFP was similar to live cells or fixed cells imaged in PBS alone (Suppl. Fig. 1).

Recording the time course of CFP-fluorescence during bleaching at the YFP-excitation wavelength interestingly showed a slight but significant increase of the CFP-signal in live cells. However, in fixed and mounted cells the CFP-fluorescence declined considerably (Fig. 1B, lower panel). This decrease could be attributed to the repeated short illumination at the CFP-excitation wavelength that was necessary to acquire the time course of CFP-fluorescence and was not caused by the illumination at the YFP-excitation wavelength. When we acquired a CFP-image followed by an immediate change to the YFP-excitation lasting for 60 sec and acquisition of a second CFP-image without any illumination at the CFP-excitation wavelength in between, we did not observe any reduction in CFP-fluorescence (data not shown). Nevertheless, the extremely fast bleaching of CFP in cells embedded in commercial mounting fluids makes it quite difficult to preserve the CFP-fluorescence during manipulation of the microscope system – and therefore to observe a potential increase of the CFP-signal in FRET samples after bleaching of YFP in mounted samples. For live cells, the opposite problem might occur based on the fact that we observed an increase in CFP-fluorescence after illumination at the YFP-excitation wavelength, even when there was no YFP expressed. This later effect might cause false positive signals and might add to the known problem of photoconverting YFP into a CFP-like fluorescent protein upon bleaching of YFP[4,5].

Since the suppliers of commercial fluorescence mounting fluids do not specify the components of their reagents in detail, it is not known, which chemical substances cause this specific differential effect on CFP- and YFP-photostability. These products, which are produced predominantly for standard immunofluorescence specimen, have usually two major components: a compound, which polymerizes so that the coverslip is stably fixed to the glass slide; and a chemical substance such as n-propyl gallate, which slows down the fading of fluorophores like FITC by inhibiting light induced oxidation. These two components might differentially alter the fluorescence properties and photostabilities of CFP and YFP. Thus, caution should be exercised, when using commercial mounting fluids for samples containing fluorescence proteins, which have a much more complicated photochemistry than low-molecular weight fluorophores. Based on these results we are not using these mounting fluids for our samples, but instead a mixture of PBS/Glycerol (1:9), where the glycerol prevents fast drying of the sample and also protects the structure of the fluorescence protein.

Moreover, our observations confirm that the FRET microscopy method of bleaching the acceptor (YFP) and monitoring an increase in donor (CFP) fluorescence is problematic on

several levels, including differential kinetics of CFP- and YFP-bleaching in different fluids and the effect of YFP-illumination on CFP-like fluorescence.

Figure legends:

Fig. 1: A) 293 cells were transfected with a CFP-YFP fusion protein (ECYFP) representing a positive FRET sample and imaged as described[6] either as live cells or after fixation and mounting. In brief, a CFP-image was acquired, followed by bleaching of YFP and acquisition of a second CFP-image. An increase in CFP-signal upon YFP-bleaching is indicated by a pseudo-colored difference image (FRET image), which appeared much more intense for live cells than for fixed, mounted cells imaged under the same conditions. B) 293 cells were transfected with either CFP- or YFP and the bleaching of the fluorescent proteins was recorded either for live cells in medium or for fixed cells after mounting in Fisher Scientific Ultramount (Ultramount) or Dako Fluorescence Mounting Fluid (Dako). Bleaching was recorded on a Zeiss Axiovert 135 microscope using continuous illumination at the respective wavelength with 50% energy of a 100W Mercury-lamp. Metamorph™ 7.5 software was used to acquire images at 5 sec intervals. Upper panel: Bleaching of CFP at CFP-excitation; middle panel: bleaching of YFP at YFP-excitation; lower panel: time course of CFP-fluorescence at continuous YFP-excitation. A software-controlled Ludl filter wheel was applied to switch from the continuous YFP-excitation briefly to CFP-excitation for capturing a CFP-signal (camera: Photometrics Coolsnap; exposure time: 50 msec). Data points are mean of n = 4.

Suppl. Fig. 1: 293 cells were transfected with CFP or YFP and bleaching was recorded in live cells in medium; in 4% paraformaldehyde-fixed cells in PBS; in fixed cells mounted with Dako Fluorescent Mounting Fluid and in fixed cells mounted in PBS/glycerol (PBS/Glyc., 1:9) as indicated.

References

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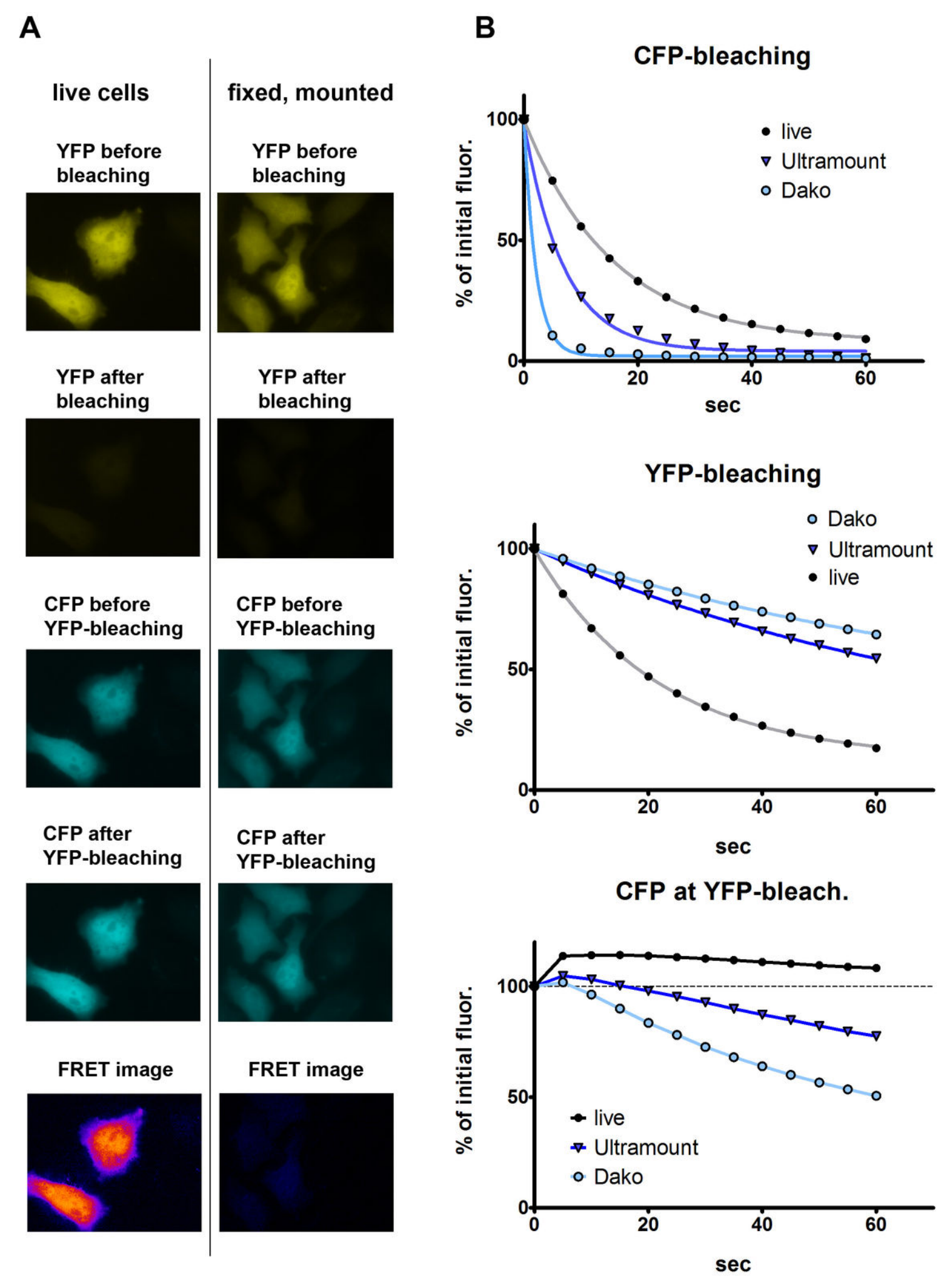
A
live cells
fixed, mounted
YFP before bleaching
YFP before bleaching
YFP after bleaching
YFP after bleaching
CFP before YFP-bleaching
CFP before YFP-bleaching
CFP after YFP-bleaching
CFP after YFP-bleaching
FRET image
FRET image
B
CFP-bleaching
% of initial fluor.
100
50
0
0
20
40
60
sec
live
Ultramount
Dako
YFP-bleaching
% of initial fluor.
100
50
0
0
20
40
60
sec
Dako
Ultramount
live
CFP at YFP-bleach.
% of initial fluor.
100
50
0
0
20
40
60
sec
live
Ultramount
Dako